Biochimica et Biophysica Acta 1853 (2015) 2481–2491

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Identification of lipid droplet structure-like/resident proteins in *Caenorhabditis elegans*

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ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form 17 May 2015
Accepted 19 May 2015
Available online 27 May 2015

Keywords:
Lipid droplet
Structure-like/Resident Proteins
Triacylglycerol (TAG)
MDT-28
Perilipin family

ABSTRACT

The lipid droplet (LD) is a cellular organelle that stores neutral lipids in cells and has been linked with metabolic disorders. *Caenorhabditis elegans* has many characteristics which make it an excellent animal model for studying LDs. However, unlike in mammalian cells, no LD structure-like/resident proteins have been identified in *C. elegans*, which has limited the utility of this model for the study of lipid storage and metabolism. Herein based on three lines of evidence, we identified that MDT-28 and DHS-3 previously identified in *C. elegans* LD proteome were two LD structure-like/resident proteins. First, MDT-28 and DHS-3 were found to be the two most abundant LD proteins in the worm. Second, the proteins were specifically localized to LDs and we identified the domains responsible for this targeting in both proteins. Third and most importantly, the depletion of MDT-28 induced LD clustering while DHS-3 deletion reduced triacylglycerol content (TAG). We further characterized the proteins finding that MDT-28 was ubiquitously expressed in the intestine, muscle, hypodermis, and embryos, whereas DHS-3 was expressed mainly in intestinal cells. Together, these two LD structure-like/resident proteins provide a basis for future mechanistic studies into the dynamics and functions of LDs in *C. elegans*.
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1. Introduction

The current upswing in research interest in lipid droplets (LDs) has been fueled by their connection to human metabolic disorders, the importance of neutral lipids in food products, and the development of biofuels [1–5]. LDs have been found in almost all organisms from bacteria to mammals and throughout most cell types in multicellular organisms [5,6]. LDs are a cellular organelle that consists of a neutral lipid core covered with a monolayer phospholipid membrane and proteins. The core contains triacylglycerol (TAG), cholesterol esters, and ether lipids [7]. LD-associated proteins have been identified in many species, from bacteria to humans [5], and can be categorized into four groups: LD structure-like/resident, lipid synthetic and metabolic, membrane traffic, and cell signaling proteins [8]. Perilipin [9] and adipose differentiation-related protein (ADRP) [10,11] are considered LD structure-like/resident proteins. They belong to the Perilipin family (PLINs), which includes three other members: Tip47 [12], S3-12 [13] and OXPAT [14]. PLIN family proteins are only expressed in mammals and *Drosophila*[15].

Further, LDs have been observed to be closely linked both at a molecular level of communication and also proximity to endoplasmic reticulum [16,17], early endosomes [18], mitochondria [19], peroxisome [20], and other cellular organelles [21], implying a possible role for LDs in energy metabolism regulation and intracellular lipid trafficking. Although LDs are an important cellular organelle and its research has significant progresses in last decade, the mechanisms behind LD formation, morphological changes and functions remain elusive.

LDs have been studied in many organisms, providing opportunities for comparative analyses. Among them *C. elegans* stands out as an excellent animal model, not only due to the ease of genetic manipulation and visualization, but also because of the demonstrated linkages between fat storage, metabolism, reproduction, and the animal's lifespan [22–26]. Our previous study provided a shotgun proteome and identified a LD marker protein DHS-3. However, the utility of this animal model for LD research has been limited due to a lack of knowledge regarding LD structure-like/resident proteins [25,27,28].

Following up on our previous study where we identified a LD marker protein, DHS-3, in *C. elegans*[29], we have performed a comprehensive

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http://dx.doi.org/10.1016/j.bbamcr.2015.05.020
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proteomic study of LDs isolated from *C. elegans*. We have identified two major LD proteins in the animal, MDT-28 and DHS-3. Both proteins were localized to LDs by fluorescence microscopy. DHS-3 was only expressed in the intestine, whereas MDT-28 was located in most tissues. We used mutational analysis to identify the regions of the proteins responsible for LD targeting. Finally, we demonstrated that the depletion of MDT-28 induces LD clustering while DHS-3 deletion reduces TAG. These data indicate that MDT-28 and DHS-3 are two LD structure-like/resident proteins in the worm, which will facilitate the study of LDs and lipid metabolism in this important animal model.

2. Materials and methods

2.1. Strains and culture conditions

The N2 Bristol strain of *C. elegans* was used as wild type in this study. The *dhs-3*(gk873395) worm was provided by the *Caenorbhabditis* Genetics Center (CGC) at the University of Minnesota. The *mdt-28*(*tm1704*) and *F22F7.1*(*tm5652*) worms were provided by National BioResource Project (NBRP). The *Pdhs-3::dhs-3::GFP*, *Pmdt-28::mdt-28::mCherry*, and *PF22F7.1::F22F7.1::GFP* worms were constructed in our laboratory. Strains *Pvha-6::dhs-3::GFP* (single copy) and *Pmdt-28::mdt-28::mRuby* (single copy) were generated by professor Ho Yi Mak. Muscle and hypodermis specific expression markers *Pmyo-3::GFP* and *Pceh-14::GFP* were crossed with *Pmdt-28::mdt-28::mCherry* to illuminate the tissue distribution of MDT-28. The *Pvha-6::dhs-3::GFP, mdt-28, Pvha-6::dhs-3::GFP, F22F7.1, Pvha-6::dhs-3::GFP, mdt-28, and F22F7.1* strains were prepared by our laboratory for the *mdt-28* and *F22F7.1* phenotype study. All worms were maintained on agar plates seeded with an OP50 bacterial lawn using a standard protocol.

The CHO K2 cell line was cultured by a method described previously [30] and used for the DHS-3 and MDT-28 lipid droplet targeting experiment.

2.2. Isolation of lipid droplets

LDs were isolated by the method previously described [29,31]. First, about 4×10^5 young adults were harvested and washed with Phosphate Buffered Saline (PBS)/0.001% Triton-X100 and suspended in 20 ml buffer A (25 mM Tricine, pH 7.6, 250 mM sucrose, and 0.2 mM phenylmethylsulfonylfluoride), followed by homogenization using a Polytron (Cole-Parmer® Labgen™ 125 and 700 Tissue Homogenizers). The homogenate was centrifuged at 1000 *g* for 30 s. The supernatant was homogenized again by nitrogen cavitation (Ashcroft Duralife Pressure Gauge) after a 15 min, 750 pounds per square inch (PSI) incubation on ice, and was then centrifuged at 1000 *g* for 10 min. 9 ml of post-nuclear supernatant (PNS), was collected and loaded into an SW40 tube. The homogenate was overlaid with 3 ml of buffer B (20 mM HEPES, pH 7.4, 100 mM KCl, and 2 mM $MgCl_2$) and was centrifuged at 12,628 *g* for 1 h at 4 °C. The LD fraction was carefully collected from top layer of the gradient and washed with 200 l buffer B 3 times. For embryonic LD isolation, the embryos were harvested using a bleach method [32]. Briefly, 4×10^5 3–4 day old adults were collected into a 15 ml tube and resuspended in a 7 ml of ddH_2O. 1 ml of 5 N NaOH and 2 ml of bleach buffer (5% solution of sodium hypochlorite) were added and then vortexed briefly. The sample was incubated at room temperature until the worms dissolved (usually 5–8 min). The sample was then centrifuged for 1 min at 1500 *g*. The supernatant was discarded and the pellet was washed 5 times. The same LD isolation procedure described above was then carried out, starting with the nitrogen cavitation.

2.3. Protein preparation and Western blot

Proteins were precipitated using 100% acetone, and were collected by centrifugation at 20,000 *g* for 10 min. Protein pellets were dissolved in 2 × SDS sample buffer at a final concentration of about 1 mg/ml for

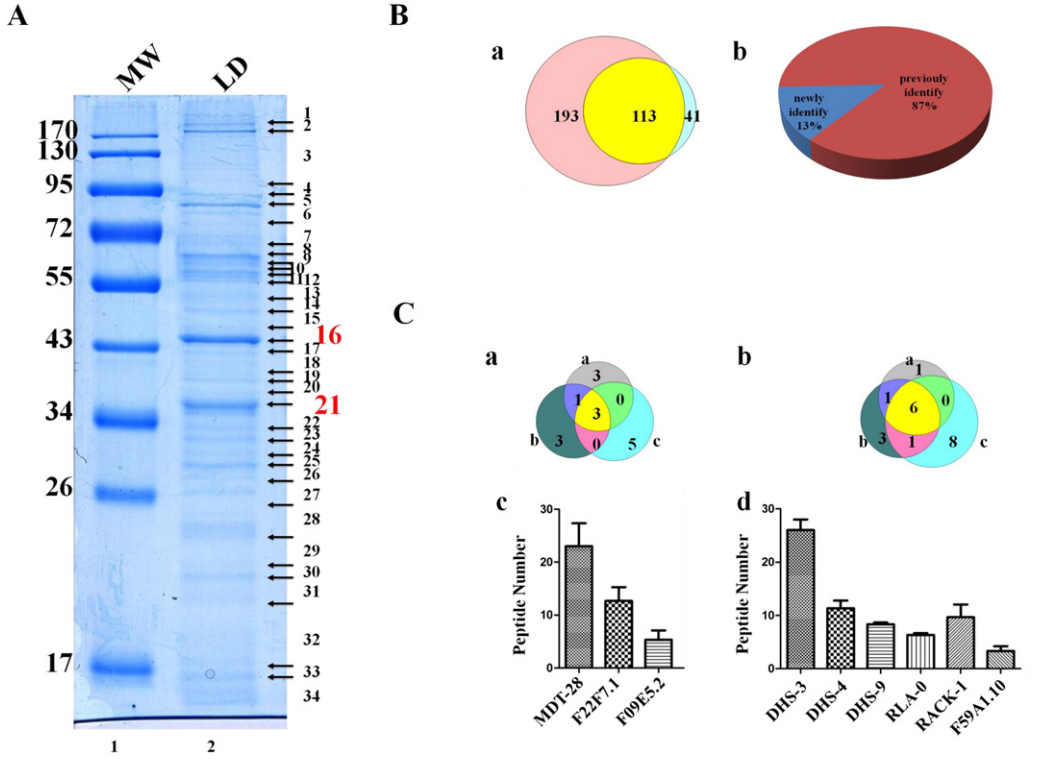


Fig. 1. Proteomic analyses of *C. elegans* lipid droplets. A. LDs were isolated from wild type adult animals, the proteins were separated by SDS-PAGE, and were stained using Colloidal blue. The LD lane was sliced into 34 pieces (arrow indicate cutting sites) and subjected to mass spectrometry protein identification as described previously [30]. B. (a) The current proteome was compared with previous LD proteomes of *C. elegans* using a Venn diagram. (b) The current proteome was compared with previous proteomic studies of the species reported except *C. elegans*. C. Proteins of the two major bands; band 16 (a) and band 21 (b) from three independent LD isolations and proteomic analyses are shown in two Venn diagrams. Peptide numbers for proteins identified in band 16 (c) and band 21 (d) are represented in the bar graphs.

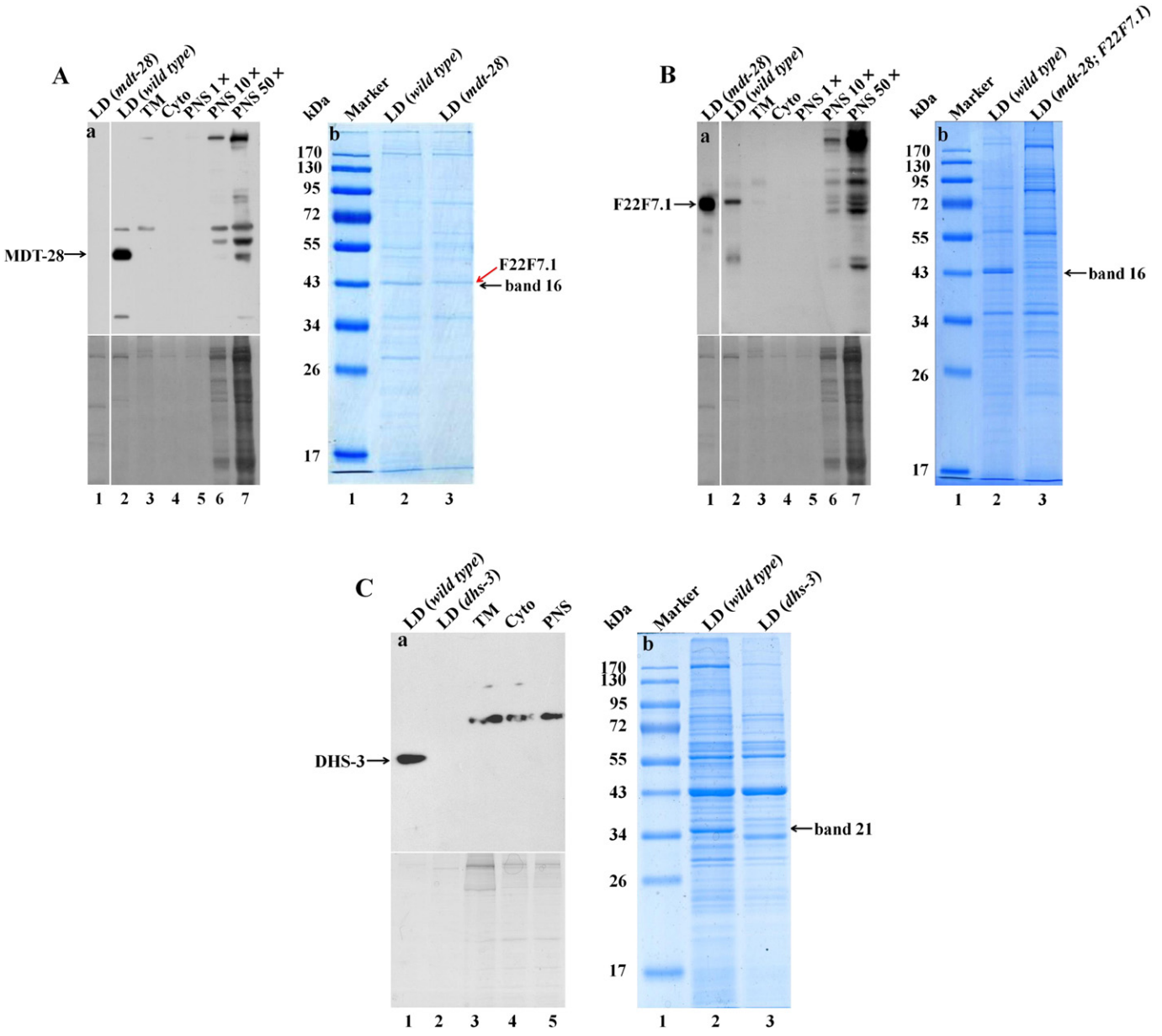


Fig. 2. Identification of lipid droplet abundant proteins. The deletion mutants of *mdt-28* and *dhs-3* were obtained, and the deletion mutant of *F22F7.1* and double mutant of *mdt-28* and *F22F7.1* were generated. LDs were isolated from all these mutants and wild type. The LD proteins were extracted and subjected to Colloidal blue staining and Western blot. A. a) MDT-28 was examined in LD, total membrane (TM), cytosol (Cyto), and postnuclear supernatant (PNS) of wild type and in LD of *mdt-28* deletion mutant using Western blot with anti-MDT-28 (upper panel). Arrow points MDT-28 band. Protein loading was detected by Coomassie blue staining (lower panel). b) LD proteins were compared between wild type and *mdt-28* deletion mutant using Colloidal blue staining. Band 16 is pointed by a black arrow and a new band is pointed by a red arrow. B. a) F22F7.1 was examined in four fractions of wild type and in LD of *mdt-28* deletion mutant using Western blot with anti-F22F7.1 (upper panel). Arrow points F22F7.1 band. Protein loading was detected by Coomassie blue staining (lower panel). b) LD proteins were compared between wild type and double mutant of *mdt-28* and *F22F7.1* using Colloidal blue staining. Band 16 is pointed by a black arrow. C. a) DHS-3 was examined in LDs of wild type and in four fractions of *dhs-3* deletion mutant using Western blot with anti-DHS-3 (upper panel). Arrow points DHS-3 band. Protein loading was detected by Coomassie blue staining (lower panel). b) LD proteins were compared between wild type and *dhs-3* deletion mutant using Colloidal blue staining. Band 21 is pointed by a black arrow.

30 min at room temperature, and were then denatured at 95 °C for 5 min. The proteins were separated by SDS-PAGE and analyzed using Western blot by a method described in our previous study [30]. Polyclonal antibodies for DHS-3, MDT-28, and F22F7.1 were prepared by AbMax Biotechnology Co., Ltd.

2.4. Mass spectrometry analysis

Lipid droplet proteins were separated on a 10% SDS-PAGE gel and subjected to colloidal-blue staining. The lane with LD proteins was cut into 34 slices. In-gel digestion of each slice was performed as follows: First, the gel was dehydrated with 100% acetonitrile and then the proteins were reduced with 10 mM DTT in 25 mM ammonium bicarbonate at 56 °C for 1 h. The proteins were then alkylated using 55 mM iodoacetamide in 25 mM ammonium bicarbonate in the dark at room temperature for 45 min. Finally, the gel pieces were thoroughly washed with 25 mM ammonium bicarbonate in water–acetonitrile (1:1, v/v) solution and were completely dried in a SpeedVac. Then proteins were incubated with 10 l trypsin solution (10 ng/ l in 25 mM ammonium bicarbonate) for 30 min on ice. 30–40 l of 25 mM ammonium bicarbonate was added after removing the excess enzyme solution. 12 hours later, 5% formic acid was added to stop the digestion reaction. A C_{18} trap column was used to capture the peptide solution, which was eluted and then subjected to nano-LC-ESI-LTQ MS/MS analysis. The LTQ mass

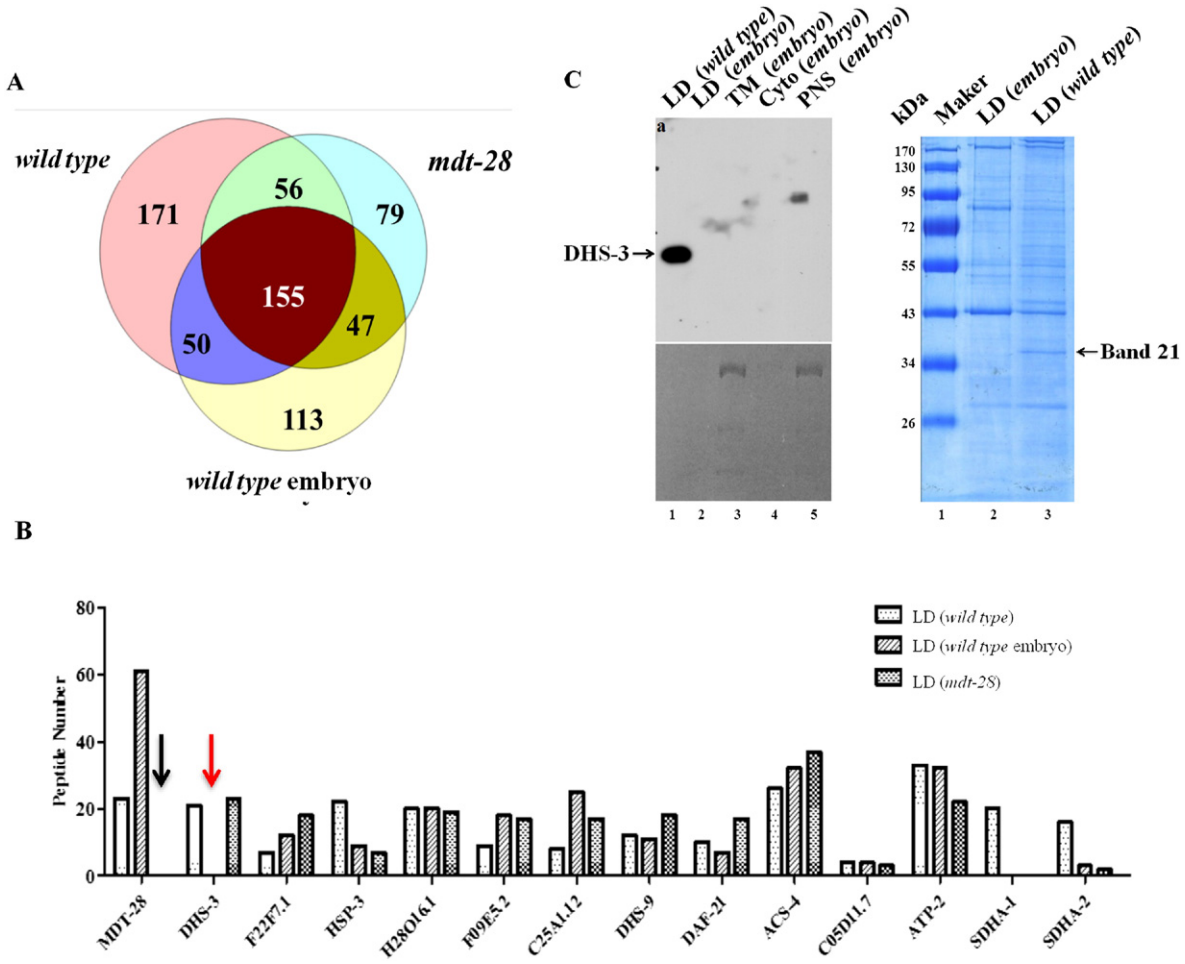


Fig. 3. DHS-3 is not expressed in embryos. A. LDs were isolated from wild type embryos and the LD proteins were subjected to proteomic analysis. The proteomes of the wild type adult and the *mdt-28* mutant were compared. B. The major proteins from proteomes of the wild type adult, the *mdt*-28 mutant adult, and wild type embryos were compared and presented with peptide numbers. C. Analysis by Coomassie blue staining and Western blot of LD proteins from wild type adults and wild type embryos. (a) Upper panel: Specific proteins in cellular fractions were examined by Western blot with polyclonal DHS-3 antibodies; lower panel: Coomassie blue-stained SDS-PAGE as a loading control. (b) LD protein profiles were presented by Colloidal blue-stained SDS-PAGE.

spectrometer was operated under data-dependent mode and was set at an initial 400–2000 Da MS scan range. The five most abundant ions were selected for subsequent collision-activated dissociation. All MS/MS data were searched against the *C. elegans* protein database Wormpep218.

2.5. Lipid droplet targeting sequences of MDT-28 and DHS-3

Following hydrophobicity and secondary structure prediction, DNA coding for DHS-3 was truncated into three fragments coding for amino acids 1–50, 50–150, and 150–307. The fragments and full length of DHS-3 were ligated into EGFP-N1, and were then transfected into CHO K2 cells. After 12 hours, the cells were harvested and fixed with 4% PFA for 30 min, permeabilized with 0.02% Triton X-100 for 8 min, and then stained with LipidTox deep red for 30 min. The prepared samples were examined using confocal microscopy. Similarly, MDT-28 was fragmented into three pieces (coding for 1–210, 210–275, 275–418 amino acids), ligated to EGFP-N1, and then transfected into the CHO K2 cells for fluorescence microscopy.

2.6. Staining and confocal microscopy

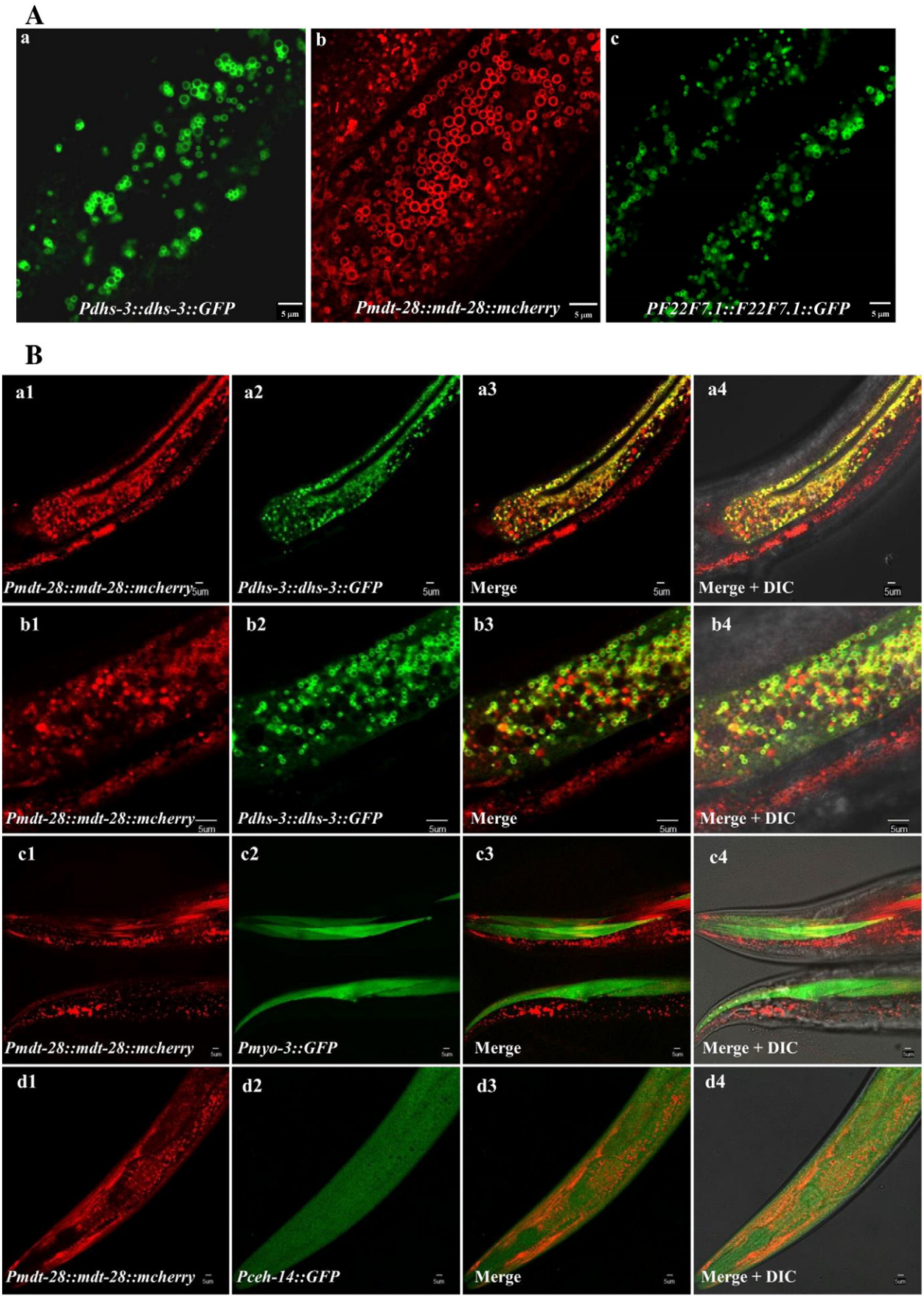
For the neutral lipid dye feeding approach, the three dyes (Nile red, Bodipy, LipidTox) were diluted 1:1000 with PBS and 200 l of the mixture was applied to an OP50 lawn in a Nematode Growth Media (NGM) plate. Then *Pdhs-3::dhs-3::GFP* L4 stage worms were transferred onto the plate. The worms were ready for live image observation after 12 hours.

Fixed Oil Red O and Nile red staining of adult worms was carried out as previously described [29,33]. The stained worms were laid on a 2% agar plate, and then subjected for confocal image analysis. For fixed Bodipy staining of embryos, we used the same protocol as for the fixed Nile red staining of adult worms.

2.7. SRS and fluorescence imaging

Stimulated Raman scattering (SRS) and fluorescence microscopy setup and imaging methods have previously been described [28]. Pump (780 nm–990 nm, tunable) and Stokes (1064 nm) laser beams

Fig. 4. Localization and tissue distribution of primary LD proteins. A. LD localization of DHS-3, MDT-28, and F22F7.1. Transgenic worms with *Pdhs-3::dhs-3::GFP* (a), *Pmdt-28::mdt-28::mcherry* (b), and *PF22F7.1-22::F22F7.1::GFP* (c), respectively, were constructed and then visualized by confocal microscopy as described previously [29]. B. Tissue distribution of MDT-28 and DHS-3. The transgenic animals co-expressing *Pmdt-28::mdt-28::mcherry* and *Pdhs-3::dhs-3::GFP* (a and b were duplicate with different magnification of microscopy), *Pmdt-28::mdt-28::mcherry* and *Pmyo-3::GFP* (c), and *Pmdt-28::mdt-28::mcherry* and *Pceh-14::GFP* (d) were generated and examined using confocal microscopy. C. Transgenic worms expressing *Pdhs-3::dhs-3::GFP* were made and visualized by confocal microscopy as described in the methods. Upper panel: GFP image; lower panel: merged picture of DIC and GFP. Bar = 5 m.


A
a
Pdhs-3::dhs-3::GFP
5 μm
b
Pmdt-28::mdt-28::mcherry
5 μm
c
PF22F7.1::F22F7.1::GFP
5 μm
B
a1
Pmdt-28::mdt-28::mcherry
5um
a2
Pdhs-3::dhs-3::GFP
5um
a3
Merge
5um
a4
Merge + DIC
5um
b1
Pmdt-28::mdt-28::mcherry
5um
b2
Pdhs-3::dhs-3::GFP
5um
b3
Merge
5um
b4
Merge + DIC
5um
c1
Pmdt-28::mdt-28::mcherry
5um
c2
Pmyo-3::GFP
5um
c3
Merge
5um
c4
Merge + DIC
5um
d1
Pmdt-28::mdt-28::mcherry
5um
d2
Pceh-14::GFP
5um
d3
Merge
5um
d4
Merge + DIC
5um